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Transcriptomic biomarkers in safety and risk assessment of chemicals

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INTRODUCTION

Identification and use of transcriptomic biomarkers to distinguish physiological conditions or clinical stages is an emerging field that has advanced substantially during the last few years. This is partly due to several national and international agencies' recommendations to incorporate advanced technologies into the safety and risk assessment process. Transcriptomics is recognized as an unbiased, sensitive, and personalized approach with the potential to reveal new predictive biomarkers of disease and ultimately improve the decision-making process. When performed in a dose−response format, the observed transcriptional changes can provide both quantitative and qualitative information on the dose at which cellular processes are affected. Transcriptomic approaches have transformed the way in which physicians approach diagnosis, prognosis, and treatment, and in which regulators approach risk assessment. This chapter provides fundamental insights into the promising technology of transcriptomics with multiple applications in both regulatory science and clinical research, where in it can be used to discover novel biomarkers potentially useful for human safety and risk assessment.

Transcriptomics

The transcriptome includes the total complement of messenger RNA (mRNA) molecules, also called "transcripts," produced in a specific cell or the population of cells comprising a tissue (Heidecker *et al.*, 2008). While transcripts originate from less than 5% of the genome in humans and other mammals, each gene (locus of expressed DNA) may produce a variety of mRNA molecules using the process of alternative splicing. Therefore, the transcriptome has a level of complexity greater than the genome that encodes it. Transcriptomics is regarded as a high-throughput technology concerned with determining how the transcriptome changes with respect to various factors at a certain time point and at a given biological state. Regulation of gene expression is highly complex and underlies many fundamental biological processes such as growth, differentiation, and disease pathogenesis with the ability to adapt rapidly with tremendous variability in different tissues and in response to stimuli (Sandvik *et al.*, 2006). Transcriptomics and global gene expression are powerful tools used in the field of toxicology and, as in most cases, toxicity is not expected to occur without alterations at the transcriptional level (Eun *et al.*, 2008; Gibb *et al.*, 2011). A common application of transcriptomics in toxicology is to compare gene expression after a chemical exposure from a diseased and nondiseased tissue to provide a list of genes that show altered expression in the diseased group. Such findings not only advance our understanding of disease pathogenesis, but they also reveal transcripts that can be qualitatively or quantitatively assessed as new biomarkers.

Within the National Institutes of Health (NIH), the term biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of

normal biologic processes, pathologic processes, or pharmacologic responses to a therapeutic intervention (Ilyin et al., 2004). Although a single biomarker can be easily understood, transcriptomic studies have shown that aggregate measures composed of multiple genes are also informative as biomarkers of complex disease. Efforts in transcriptomic biomarker development are not restricted to medical diagnostics but include environmental chemical risk assessment and determination of exposure to chemical residues in food (Riedmaier et al., 2009a,b,c; Pinel et al., 2010).

Transcriptomic methods

There are three commonly used methods for assessing transcriptomes: quantitative real-time polymerase chain reaction (qPCR), microarrays, and RNA sequencing (RNAseq). Each of these target methods has advantages and disadvantages which can be further subdivided into targeted or untargeted approaches. The qPCR is a targeted method that uses short DNA sequences called primers to anneal and amplify known transcripts in a biological sample. Rapid quantitation of hundreds of transcripts using qPCR is relatively inexpensive, but knowledge of sequences of the candidate transcripts is required. The two main technologies for untargeted global transcriptome screening that dominate the diagnostic field are gene expression microarrays and RNAseq. In microarray-based methods, RNA is isolated from a specific sample and converted to a chemically labeled form. Labeled RNA is then incubated with a small chip that measures transcript abundance by hybridization of labeled RNA to each of the probes on the microarray. Microarrays offer genome-wide coverage of the transcriptome, have high throughput, and have become relatively inexpensive but complicated normalization methods and, similarly to qPCR, are limited by the requirement to know existing transcript sequences up front. Despite these limitations, microarrays have thus far been the most widely applied technology. RNAseq is the most recent transcriptomic approach where the total complement of RNAs from a given sample is isolated and sequenced using high-throughput technologies (often called Next-Generation Sequencing). The abundance of each transcript is quantitated by counting the number of copies. RNA sequencing has the advantage, as compared to microarrays and qPCR, in that no previous knowledge on sequence is necessary. However, RNAseq methods are costly, and complex and require enormous computational capacity. In some situations, RNAseq has already begun to replace microarrays in basic research, but clinical studies will likely use both approaches depending on scientific goals, sample size, and cost. Looking forward, it is clear that advances in our fundamental understanding of gene transcription and rapidly advancing techniques for transcriptome assessment will have a continued impact on biomarker development. However, one hurdle in the field is to establish better platforms for the reproducibility and predictive accuracy between sites (Fielden et al., 2008).

Types of transcriptomic biomarkers

There are three commonly used types of transcriptomic biomarkers: messenger RNA (mRNA), micro RNA (miRNA), and long noncoding RNA (lncRNA). mRNA biomarkers are already an established method in several scientific fields. Pathogenic tissue can be distinguished from nonpathogenic by analyzing the expression of specific genes. Using mRNA gene expression analysis is helpful in validating different types or stages of diseases. Noncoding RNAs can be subdivided into small noncoding RNAs shorter than 200 nt and long noncoding RNAs with more than 200 nt (Gibb et al., 2011). miRNAs are small noncoding RNA molecules with about 20−22 nucleotides which are involved in posttranscriptional processing of mRNA. In this way they are able to regulate physiological pathways and metabolic processes and therefore impact the entire cellular physiology, organ development, and tissue differentiation. The expression of miRNAs can be measured in cell culture samples. These miRNAs are also present in body fluids, such as urine, blood, and breast milk (Laterza et al., 2009; Kosaka et al., 2010; Kroh et al., 2010). Noncoding RNAs with a length of more than 200 nt belong to the group of long noncoding RNAs (lncRNAs) and have been identified as biomarkers in several disease conditions. Interestingly, the identification of single biomarkers on the mRNA or the miRNA level is not possible in most pathological disorders. In such cases, a set of multiple biomarkers must be present to distinguish between specific disease types, disease states, or applied treatments. Bioinformatics is one approach that can be used to integrate the data analysis of multiple biomarker levels of different types of transcriptomic biomarkers (i.e., mRNA, miRNA, and lncRNA). This could help to generate an integrative gene expression pattern (Molloy et al., 2011). To achieve this goal, different multivariate analysis methods are available which are used for biomarker selection and validation, namely hierarchical cluster analysis (HCA) and principal components analysis (PCA).

TRANSCRIPTOMICS IN BIOMARKER DISCOVERY

Transcriptomic approaches have been used by various researchers to identify novel biomarkers, which are

beginning to influence both clinical practice and environmental chemical risk assessment. This section focuses on biomarkers of cardiovascular disease and hepatotoxicity, exposure to anabolic steroids and beta-agonists, and the use of biomarkers in pathway analysis for quantitative environmental chemical risk assessment.

Cardiovascular biomarkers

Cardiovascular disorders are responsible for high morbidity and mortality and pose a substantial economic burden at the individual, institutional, and national levels. The use of the human cardiac transcriptome as a biomarker to improve clinical diagnosis has been slow to develop, mostly due to increased risks and complications associated with endomyocardial biopsies (Zhang et al., 2003). However, recent transcriptomic experiments using human cardiac biopsies have been conducted and used to identify specific causes of cardiomyopathy, (Farazi et al., 2011) including subtypes of myocarditis (Soga et al., 2002). The ST2 protein, encoded by the IL1RL1 gene, is markedly elevated in patients with heart failure and exists in a soluble form that can be measured in peripheral blood. ST2 was first identified as a potential biomarker upregulated in an in vitro transcriptomics study involving myocardial stretch. Follow-up studies in animals have suggested that ST2 is part of a cardioprotective paracrine signaling axis between cardiac fibroblasts and myocytes (Sanada et al., 2007; Kakkar and Lee, 2008). ST2 signals the presence and severity of adverse cardiac remodeling and tissue fibrosis, which routinely occurs in response to myocardial infarction, acute coronary syndrome, or worsening heart failure. ST2 use as a biomarker has considerable prognostic value and is used as an aid for risk stratification in identifying patients who are either at high risk of mortality or need rehospitalization. As a result of these biomarker discovery studies, a commercial-grade soluble ST2 assay for use in assessing prognoses of chronic heart failure has been cleared by the US FDA (Presage, Critical Diagnostics, San Diego, CA). Although the ST2 immunoassay measures protein levels in the blood, the use of transcriptomics was vital in its discovery. The initial findings of the transcriptomic screens have led to both a novel cardiac biomarker and therapeutic target for heart failure.

The use of miRNAs as a cardiovascular disease biomarker is also promising. While most of the evidence is limited and further work in larger human populations is required for validation, the evidence demonstrates the feasibility of using miRNAs as biomarkers. Associations between miRNAs and cardiovascular endpoints include acute coronary syndrome (Widera et al., 2011), acute myocardial infarction (Ji et al., 2009; Wang et al., 2009; Corsten et al., 2010; D'Alessandra et al., 2010), hypertension (Li et al., 2011), and heart failure (Latronico et al., 2007; Tijsen et al., 2010).

Hepatic biomarkers

Several studies have used transcriptomics to identify biomarkers of hepatotoxicity and to determine their mechanism-of-action (Kussmann et al., 2006; Mutlib et al., 2006; Nie et al., 2006; Tugendreich et al., 2006; Fielden et al., 2007; Eun et al., 2008). Liver gene expression profiling by microarray technology was used to develop biomarkers for nongenotoxic hepatocarcinogens (Fielden et al., 2007). Fielden et al. (2007) collected hepatic gene expression data from rats treated for 5 days with 47 test chemicals. It was observed that this short-term in vivo rodent model was more sensitive, more accurate, and provided quicker results when compared with the traditional models for risk assessment of nongenotoxic carcinogens. qRT-PCR has also been used for the identification of biomarkers of toxicity (Ellinger-Ziegelbauer et al., 2009). These studies and several others in the open literature demonstrate that transcriptomic technologies are valuable, sensitive tools for screening hepatotoxic as well as hepatocarcinogenic potential of suspected test agents, although their biomarkers and mechanisms of action may be different.

Biomarkers of anabolic agents

Screening for anabolic agents such as steroid hormones (testosterone or estrogen) and beta-agonists in meat-producing animals demonstrates an application of transcriptomics for use in safety and regulatory monitoring. In agricultural meat-producing animals, the myotropic, growth-promoting properties of steroid hormones and beta-agonists are beneficial because of increased productivity and reduced costs with weight gain and feed efficiency (Chwalibog et al., 1996). As a consequence, hormone and drug residues in meat are increased and adverse side effects to the consumer occur (Daxenburger et al., 2001; Lange et al., 2001; Maume et al., 2001; Swan et al., 2007). Questions and controversy over the impacts of these added hormones on human development and health have lingered for decades. The use of growth promoters is approved in the US, Canada, Mexico, Australia, and South Africa, and although the USFDA currently does not regulate their use in cattle, these drugs are prohibited for over-the-counter use by humans. The European Union (EU) forbids the use of these substances, including the importation of meat from animals treated with them. To enforce the EU rules, the monitoring of anabolic agents in meat is necessary.

Current methods to uncover the abuse of anabolic agents in animal residues are limited to immune assays or chromatographical methods (Mayer et al., 1991; Mayer et al., 2011), which can only detect known substances. The use of transcriptomics is a promising approach to develop a biomarker pattern based on physiological changes that result after illegal application of anabolic agents. Discovery of a single transcription biomarker in cattle for a particular organ or tissue is challenging; therefore, gene expression profiles, the measurement of several genes at one time, is used (Riedmaier et al., 2009a,b,c). Promising candidate genes and gene profiles for the development of a biomarker screening method in cattle include insulin-like growth factor 1 (IGF-1) in liver and muscle (Johnson et al., 1998; Pfaffl et al., 2002; Reiter et al., 2007), steroid hormone receptors in the liver, muscle, uterus, gastrointestinal tract, kidney, prostate, and blood cells (Pfaffl et al., 2002; Toffolatti et al., 2006; Reiter et al., 2007; Riedmaier et al., 2009a,b,c), and various inflammatory, apoptotic and proliferative genes in blood cells (Chang et al., 2004; Cantiello et al., 2007; Riedmaier et al., 2009a,b,c). Agonists for the beta-andrenergic receptor are known to affect mRNA expression of different muscle proteins, such as α-actin, myosin, or calpastatin in cattle. Overall, the use of transcriptomics and other omics technologies can be used to develop new screening methods for the detection of the misuse of anabolic steroids and beta-agonists for public health protection.

Environmental chemical risk assessment

The US EPA, the National Toxicology Program (NTP), and the National Research Council recommend the use of pathway analysis as a basis for toxicity testing and chemical risk assessment (Collins, 2008; Thomas et al., 2012). To date, the application of transcriptomic data in risk assessment has focused mostly on elucidating a chemical's mechanism or mode-of-action (Thomas et al., 2012). Methods to apply transcriptomics in a quantitative risk assessment are currently in their infancy with no national or international agency-derived risk values. The challenge is partly due to the translatability of the RNA or pathway change on the toxicity endpoint. To determine whether a pathway-based transcriptomic approach leads to quantitative differences in their relationship to apical endpoints, Thomas et al. (2012) reanalyzed previous dose-response changes in gene expression to identify potential toxicity pathways that underlie chemically induced noncancer and cancer endpoints in the mouse lung and liver. Female B6C3F1 mice were previously exposed to multiple concentrations of five chemicals (1,4-dichlorobenzene, propylene glycol mono-t-butyl ether, 1,2,3-trichloropropane, methylene chloride, and naphthalene) that had tested positive for lung or liver tumor formation in two-year rodent cancer bioassays conducted by the NTP (Thomas et al., 2011). The dose−response changes in gene expression were analyzed using a standard benchmark dose modeling (BMD) approach, and then grouped based on cellular/biological processes. The transcriptional points of departure for the five chemicals were compared to both cancer and noncancer points of departure based on apical endpoints. Overall, the transcriptomic responses corresponded closely with both noncancer and cancer apical responses as a function of dose, and can be used as points-of-departure. Transcriptomic changes in canonical pathways are a promising approach for identification of biomarkers to estimate the noncancer and cancer points-of-departure for use in quantitative risk assessments. Transcriptomic methods can also be applied to support the use of a particular study over another for the derivation of a reference value (RfV). Szabo et al. (2013) conducted a whole animal microarray study on the hippocampus of C57BL/6 mice orally exposed to hexabromocyclododecane (HBCD), a brominated flame retardant currently going through an EPA human health risk assessment. The results identified canonical pathways which support neurobehavioral endpoints (Szabo et al., 2013) The high correlation between these pathways and rodent toxicity indicates the need for further investigation of their role in these endpoints and examination of their relevance to human responses. If they are relevant, these pathways could be considered toxicity pathways and used as the basis for future high-throughput toxicity screening.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Transcriptomics as a discipline is still evolving and, although there are no clearly defined methods for the discovery of biomarkers using gene expression technologies, the potential of this new field seems promising. There is growing demand for transcriptional biomarker development in a variety of applications, including clinical diagnosis, pathological processes, biomarkers for environmental chemical exposure or response, and safety evaluation. The discovery of predictive markers that precede pathology should be able to lessen adverse impact on health. Since gene transcription is a very dynamic process able to adapt rapidly to environmental, physiological, or pathological changes, the transcriptome is a preferred data set for the identification of sensitive and predictive biomarkers. It is conceivable that both a single biomarker and the concept of multimarker panels will become the standard in biomarker research

(Ky *et al.*, 2011). To validate an identified transcribed biomarker set with high significance, the application of bioinformatical tools is necessary. Considering that there are different levels at which biomarkers can be measured and evaluated, and that technologies continue to advance, new categories of transcriptomic biomarkers will continue to emerge.

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